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Genetic background affects the expansion of macrophage subsets in the lungs of *Mycobacterium tuberculosis*-infected hosts

Thais Barboza Bertolini,[1,*] Alexandre Ignacio de Souza,[1,*] Ana Flávia Gembre,[1] Annie Rocio Piñeros,[1] Rafael de Queiroz Prado,[1] João Santana Silva,[1] Leandra Naira Zambelli Ramalho[2] and Vânia Luiza Deperon Bonato[1]

[1]*Department of Biochemistry and Immunology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo, and* [2]*Department of Pathology, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo, Brazil*

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*These authors contributed equally to this work.
Correspondence: Professor Vânia L. D. Bonato, Ribeirão Preto Medical School, University of São Paulo, Ribeirão Preto, São Paulo. Av. Bandeirantes, 3900, Ribeirão Preto, São Paulo. 14049-900, Brazil.
Email: vlbonato@fmrp.usp.br
Senior author: Vânia Luiza Deperon Bonato

Summary

M1 macrophages are more effective in the induction of the inflammatory response and clearance of *Mycobacterium tuberculosis* than M2 macrophages. Infected C57BL/6 mice generate a stronger cellular immune response compared with BALB/c mice. We hypothesized that infected C57BL/6 mice would exhibit a higher frequency and function of M1 macrophages than infected BALB/c mice. Our findings show a higher ratio of macrophages to M2 macrophages in the lungs of chronically infected C57BL/6 mice compared with BALB/c mice. However, there was no difference in the functional ability of M1 and M2 macrophages for the two strains *in vitro*. *In vivo*, a deleterious role for M2 macrophages was confirmed by M2 cell transfer, which rendered the infected C57BL/6, but not the BALB/c mice, more susceptible and resulted in mild lung inflammation compared with C57BL/6 mice that did not undergo cell transfer. M1 cell transfer induced a higher inflammatory response, although not protective, in infected BALB/c mice compared with their counterparts that did not undergo cell transfer. These findings demonstrate that an inflammation mediated by M1 macrophages may not induce bacterial tolerance because protection depends on the host genetic background, which drives the magnitude of the inflammatory response against *M. tuberculosis* in the pulmonary microenvironment. The contribution of our findings is that although M1 macrophage is an effector leucocyte with microbicidal machinery, its dominant role depends on the balance of M1 and M2 subsets, which is driven by the host genetic background.

Keywords: genetic background; inflammation; inducible nitric oxide synthase; M1 macrophages; M2 macrophages; tuberculosis.

Introduction

Tuberculosis kills 1·5 million people worldwide each year.[1] Despite huge progress in understanding the activation of the different leucocyte populations and mediators that participate in host protection, the immune response against *Mycobacterium tuberculosis* infection is complex and has not been completely deciphered. The complexity of the immune response contributes to the difficulty in developing vaccines that are more effective than bacillus Calmette–Guérin and treatment strategies based on immunotherapy.[2,3]

Susceptibility to tuberculosis is attributed to multiple factors but is predominantly dependent on host genetic and environmental factors.[4,5] In susceptible hosts, who comprise approximately 10% of infected people, the persistence of the bacillus confers a chronic disease characterized by type IV hypersensitivity and granuloma formation.[6]

Macrophages play a key role in the induction of the immune response against *M. tuberculosis*. These cells are among the first to sense the bacillus in the lung. The phagocytosis of mycobacteria by lung macrophages generates an inflammatory response and antigen presentation to effector T cells.[7] Additionally, macrophages have several mechanisms that can kill phagocytosed bacteria and play an essential role in tissue repair as a consequence of the inflammatory process.[8]

In the steady-state condition, lung macrophages are non-inflammatory.[9,10] However, after *M. tuberculosis* infection, different pattern recognition receptors expressed by macrophage and dendritic cell subsets sense

mycobacterial pathogen-associated molecular patterns and induce the secretion of tumour necrosis factor, interleukin-1β (IL-1β), IL-12, IL-6, IL-23 and chemokines through MyD88 and nuclear factor-κB expression.[11] Hence, macrophages cooperate to drive the magnitude of the inflammatory response and the pattern of the adaptive immune response. Depending on the density of virulence factors, pattern recognition receptors and pathogen-associated molecular patterns, infected macrophages and dendritic cells may also produce anti-inflammatory mediators such as IL-10 that contribute to the down-modulation of the effector functions of antigen-presenting cells and interferon-γ (IFN-γ) -producing $CD4^+$ cells.[12] The balance between the pro- and anti-inflammatory responses is crucial for the tolerance of infection mediated by IFN-γ-dependent mechanisms that induce bacterial clearance and the tolerance to tissue damage, which protects the host against excessive type IV hypersensitivity.

During experimental infection with *M. tuberculosis*, the deficiency of any component that disturbs the induction of IFN-γ production and the effector mechanisms up-regulated by IFN-γ [i.e. inducible nitric oxide synthase (iNOS) expression] has been associated with progression of the infection.[13–16] As *M. tuberculosis* infection progresses, there is a change in the pattern of the inflammatory response that results in an accumulation of foamy macrophages and M2 macrophages.[17,18] M2 macrophages or alternatively activated macrophages are associated with tissue repair.[19,20] The hallmark of M2 macrophages is the expression of arginase 1 (Arg1) among other receptors; Arg1 is directly associated with an anti-inflammatory response and weak microbicidal ability.[21,22] Therefore, M2 macrophages have been associated with progression of the infection.[23–25] In contrast, M1 or classically activated macrophages express iNOS, produce nitric oxide (NO) and secrete pro-inflammatory cytokines. Hence, M1 macrophages are associated with microbicidal potential.[20] However, an unbalanced type 1 response [T helper type 1 (Th1) cells and M1 macrophages] is also detrimental for the chronically infected host.[26]

C57BL/6 mice infected with *M. tuberculosis* have been reported to generate a strong production of IFN-γ compared with infected BALB/c mice, although the bacterial load is similar between the mouse strains.[10,27] We showed that a reduction in IFN-γ production in the lungs of BALB/c mice during the chronic phase of the infection (70 days) was followed by augmentation of the IL-10 levels and $Foxp3^+$ cells compared with BALB/c mice infected for 30 days. Moreover, we observed an increase in the suppressor activity of spleen $CD4^+$ $CD25^+$ $Foxp3^+$ cells compared with C57BL/6 mice infected for 70 days.[27,28] However, we showed that chronically infected BALB/c mice were more susceptible to *M. tuberculosis* infection than C57BL/6 mice.[27] Our findings and those from other groups show that C57BL/6 mice exhibit a higher production of IFN-γ than BALB/c mice; this increased production is independent of the ability to better control lung bacterial replication.[10,27] Recently, we described how $CD11c^+$ $CD103^+$ cells, which were found at a high frequency in the lungs of infected C57BL/6 mice but not infected BALB/c mice (which presented a high frequency of $CD11c^+$ $CD11b^+$ cells), were associated with a high frequency of IFN-γ- or IL-17-producing $CD4^+$ cells.[29]

In the present study, our aim was to evaluate macrophage subsets in the lungs of *M. tuberculosis*-infected C57BL/6 and BALB/c mice. The differences in the immune responses conferred by the genetic background may assist in defining biomarkers associated with protective or deleterious immune responses and the magnitude of inflammation. We hypothesized that infected C57BL/6 mice would exhibit a higher frequency and function of M1 macrophages, whereas infected BALB/c mice would exhibit a higher frequency of M2 macrophages; this differential pattern of macrophage subset expression would affect the progression of the infection.

Material and methods

Animals

Specific pathogen-free female C57BL/6 and BALB/c mice (6–8 weeks old) were obtained from the local breeding facility of the Ribeirão Preto Medical School, University of São Paulo (FMRP-USP), Brazil. All animals were maintained under barrier conditions in a level III biosafety laboratory with free access to sterile food and water. All procedures were approved by the local research ethics committee of FMRP-USP (protocol number 088/2014).

Bacteria and infection

The H37Rv *M. tuberculosis* strain (American Type Culture Collection 27294, Rockville, MD) was grown in 7H9 Middlebrook Broth (DIFCO Laboratories, Detroit, MI) for 7 days at 37°. The culture was harvested as previously described.[30] The bacterial suspension was diluted in PBS and adjusted according to the number 1 McFarland scale.[30] The mice were anaesthetized by intraperitoneal injection of 100 µl of a saline solution containing 20% ketamine (Ketamina Agener; Embu-Guaçu, SP, Brazil) and 10% xylazine (Dopazer®; Laboratorios Calier S.A., Barcelona, Spain) and infected by administration of 1×10^5 bacilli by the intratracheal route.[31] The lungs were evaluated at 30 or 70 days post-infection.

Colony-forming units assay

The lower and middle right lobes of the lungs and the spleen were washed with sterile PBS. Each tissue was

placed in a Petri dish containing incomplete RPMI-1640 medium (Sigma, St Louis, MO) and processed as previously reported.[29] For determination of colony-forming units (CFU), serial dilutions (100, 1000, 10 000 and 100 000) of the digested lungs and serial dilutions (10, 100, 1000 and 10 000) of the digested spleens were plated onto supplemented 7H11 agar media (Difco, Becton, Dickinson and Company, Le Pont de Chaix, France). The CFU number was counted after 28 days of incubation at 37°, and the results were expressed as the $\log_{10}$ of the CFU/g of the lung and the $\log_{10}$ of the CFU/spleen.

Processing of lung cells

The cell suspensions obtained after lung digestion were passed through a 100-μm Cell Strainer (BD Biosciences, Becton Circle, Durham, NC), pelleted by centrifugation for 10 min at 400 ***g*** and resuspended in complete RPMI-1640 medium (10% fetal bovine serum, 10 μg/ml gentamicin, 100 U/ml penicillin and 100 μg/ml streptomycin). Total cell counts were determined in a Countess™ automated cell counter (Invitrogen, Eugene, OR).

Flow cytometry

Lung cells were initially incubated for 40 min at 4° with the supernatant of the 2.4G2 cell lineage (containing an anti-FcγRII/III antibody), followed by incubation with a monoclonal antibody (0·5 μg/1 × 10^6 cells) for 30 min at 4° in total darkness. To characterize the macrophage subsets, the following anti-mouse monoclonal antibodies were used: anti-F4/80 (clone BM8; eBioscience, San Diego, CA), anti-CD11c (clone HL3), anti-CD11b (clone M1/70) and anti-CD206 (clone C068C2) (all from BD Biosciences, San Diego, CA). Lung cells were acquired by flow cytometry using the BD FACSCanto II (BD Bioscience, Franklin Lakes, NJ). One hundred thousand events per sample were collected and analysed according to size, granularity and fluorescence intensity using the FLOWJO 7.6.1™ software (Tree Star, Inc., Ashland, OR).

Real-time PCR

Total RNA was isolated and purified from the lung using an illustra RNAspin Mini RNA Isolation Kit (GE Healthcare, Little Chalfont, UK) according to the manufacturer's instructions. The RNA was quantified in a NanoDrop™ 1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA), and cDNA synthesis was performed using the Reverse Transcriptase SuperScript™ II (Invitrogen, Life Biotechnologies, Carlsbad, CA). Real-time PCR was performed using the Maxima SYBR Green/ROX qPCR Master Mix (2×) (Thermo Fisher Scientific, Inc.) in the StepOnePlus™ Real-Time PCR System (Applied Biosystems, Foster City, CA). The samples were amplified using the following conditions: initial denaturation at 95° for 10 min, followed by 40 cycles at 95° for 15 seconds, an annealing phase at 58° for 30 seconds and an extension phase at 72° for 30 seconds. The samples were analysed using the C_t (Threshold Cycle) method. Gene expression was calculated as $2^{-(\Delta\Delta C_t)}$, where $\Delta\Delta C_t = \Delta C_t$ (sample) – ΔC_t (calibrator) and ΔC_t (sample) = C_t (target gene) – C_t (normalizer = β-Actin). The following primer sequences were used: *β-Actin* (forward, 5′-CCC TAG GCA CCA GGG TGT GA-3′ and reverse, 5′-GCC ATG TTC AAT GGG GTA CTT C-3′), *iNOS* (forward, 5′-TGC TGT TCT CAG CCC AAC AAT A-3′ and reverse, 5′-GTC CAG GGA TTC TGG AAC ATT CT-3′) and *Arg1* (forward, 5′-CAA AAG GAC AGC CTC GAG GAG-3′ and reverse, 5′-CCC GTG GTC TCT CAC GTC AT-3′).

Immunohistochemistry

Sections of deparaffinized lung samples were subjected to antigenic recovery by incubation for 15 min at 42° in citrate buffer (1.5 g of $C_6H_8O_7{\cdot}H_2O$ in 500 ml of distilled water, pH 6·0), followed by washing with PBS containing 0·01% saponin for 3 min and incubation overnight at 4° with the primary antibody (rabbit anti-mouse NOS2, sc_650; Santa Cruz Biotechnology, Heibelberg, Germany) diluted 1 : 400 in PBS/0.01% saponin. The secondary biotinylated antibody (biotinylated goat anti-rabbit IgG) and streptavidin (Vector Laboratories, Burlingame, CA) conjugated to peroxidase were used to improve the sensitivity of the assay. The reaction was visualized by incubating the sections with 3,3-diaminobenzidine tetrahydrochloride (DAB; Zymed Laboratories, Inc., San Francisco, CA). Control slides were incubated with secondary antibody. Counterstaining with haematoxylin was performed to visualize the nuclei, and the images were acquired as described in the histological analysis procedures.

Bone marrow-derived macrophages

Bone marrow precursors were incubated with RPMI-1640 medium containing 10% fetal bovine serum and 30% L929 fibroblast supernatant (derived from C3H mice) in Petri plates (BD OPTILUX). The culture medium was replaced on day 3. After 7 days, 1 × 10^7 bone-marrow-derived macrophages were plated under conditions for M1 or M2 macrophage polarization using 200 ng/ml IFN-γ (R&D Systems, Minneapolis, MN) and 1 μg/ml lipopolysaccharide (Sigma-Aldrich, St Louis, MO) or 30 ng/ml IL-4 (R&D Systems), 30 ng/ml IL-13 (R&D Systems) and 30 ng/ml IL-33 (R&D Systems), respectively, for 48 hr.

Phagocytosis and killing assays

M1 and M2 macrophages (2·5 × 10^5) were plated in 24-well plates (Corning, New York, NY); after 4 hr, the cells

were infected with *M. tuberculosis* (multiplicity of infection 5). To assess phagocytosis, 4 hr after *in vitro* infection the culture medium was replaced with RPMI-1640 containing 10% fetal bovine serum without antibiotics. To assess killing, the cells were cultured for 24 hr. At the indicated time-points after 4 or 24 hr, the cells were washed and lysed with 0·05% saponin. The cell lysates were serially diluted in PBS and 100 μl were plated on Middlebrook 7H11 agar. The CFU counts were determined after 28 days of incubation at 37° in 5% CO_2.

Cell transfer

Sixty days post-infection, C57BL/6 or BALB/c mice received 2×10^6 M1 or M2 macrophages differentiated from the bone marrow of their respective counterparts by the intratracheal route. As an experimental control, groups of mice also received M0 macrophages. The CFU assay and histopathological analysis were performed 10 days after the adoptive cell transfer.

Histopathology analysis

The upper right lobes of the lungs were fixed in 3·7% formaldehyde, embedded in paraffin blocks and sectioned for light microscopy. For the histopathological analysis, 5 mm sections were stained with haematoxylin & eosin.

Statistical analysis

The data were analysed for homogeneity using Levene's test followed by normality analysis with the Kolmogorov–Smirnov test and Shapiro–Wilk's test. The data were analysed using one-way analysis of variance. The Tukey's test was used for homogeneous data with a normal distribution (parametric data). The Kruskal–Wallis test was used for non-homogeneous data or data that did not assume a normal distribution (non-parametric data). The data were analysed with StatSoft Inc. (2004) STATISTICA software, version 7 (Tulsa, OK). The data were expressed as the mean ± standard deviation (SD). Values of $P < 0{\cdot}05$ were considered significant. The correlation analysis was determined by non-parametric correlation with Spearman's correlation coefficient.

Results

Chronically infected C57BL/6 mice exhibit a higher M0 : M2 ratio in the lungs than BALB/c mice

First, we confirmed previous results by evaluating the CFU numbers in the lungs and spleens of infected C57BL/6 and BALB/c mice during the initial (30 days) and chronic (70 days) phases of infection. Figure S1(a) (see Supplementary material) shows that C57BL/6 (black bars) and BALB/c (white bars) mice exhibited similar CFU counts in the lungs at 30 and 70 days post-infection. However, the CFU numbers in the lungs of the C57BL/6 mice were significantly lower than the numbers recovered from the lungs of the BALB/c mice during the chronic phase of infection (see Supplementary material, Fig. S1a). We observed the same phenomenon in the spleens (see Supplementary material, Fig. S1b). Moreover, the CFU recovery in the spleens between 30 and 70 days of infection showed the progression of the infection in BALB/c mice (see Supplementary material, Fig. S1b).

To evaluate macrophage subsets, we used the markers CD11c, CD11b, F4/80 and CD206. Macrophages (M0) were characterized as $CD11c^+$ $CD11b^+$ $F4/80^+$ cells (Fig. 1a). The M2 macrophage subset was characterized as CD206-expressing macrophages ($CD11c^+$ $CD11b^+$ $F4/80^+$) (Fig. 1a). CD206 is the mannose receptor and is highly expressed on the cell surfaces of M2 macrophages.[32] Although the frequency of macrophages (M0) was similar in the lungs of the non-infected (CT group) and infected C57BL/6 mice, there was a significant decrease in the frequency of $CD11c^+$ $CD11b^+$ $F4/80^+$ cells in the lungs of BALB/c mice 30 and 70 days post-infection compared with the non-infected counterpart group (Fig. 1b).

We found a significantly lower frequency of $CD11c^+$ $CD11b^+$ $F4/80^+$ $CD206^+$ cells in the lungs of C57BL/6 mice 30 and 70 days post-infection compared with their control counterpart group (Fig. 1c). In contrast, there was no difference in the frequency of these cells in the lungs of BALB/c mice 70 days post-infection and their respective control group, although the frequency of M2 macrophages was lower in the lungs of BALB/c mice 30 days post-infection (Fig. 1c).

To better compare these results, we analysed the ratio of macrophages (M0) to M2 macrophages (M2). Figure 1(d) shows a significant M0 : M2 ratio in the lungs of C57BL/6 mice during the chronic phase of infection compared with BALB/c mice at the same time-point. Moreover, the M0 : M2 ratio was lower when we compared BALB/c mice 30 and 70 days post-infection.

We also counted the $CD11c^+$ $CD11b^+$ $F4/80^+$ and $CD11c^+$ $CD11b^+$ $F4/80^+$ $CD206^+$ cells in the lungs of both mouse strains at 30 and 70 days post-infection. There was a significantly higher number of $CD11c^+$ $CD11b^+$ $F4/80^+$ cells in the lungs of C57BL/6 mice 30 and 70 days post-infection compared with the non-infected group. The same result was observed in the lungs of the BALB/c mice (see Supplementary material, Fig. S2a). In addition, there was an increase in the number of $CD11c^+$ $CD11b^+$ $F4/80^+$ cells in the lungs of C57BL/6 mice 30 and 70 days post-infection compared

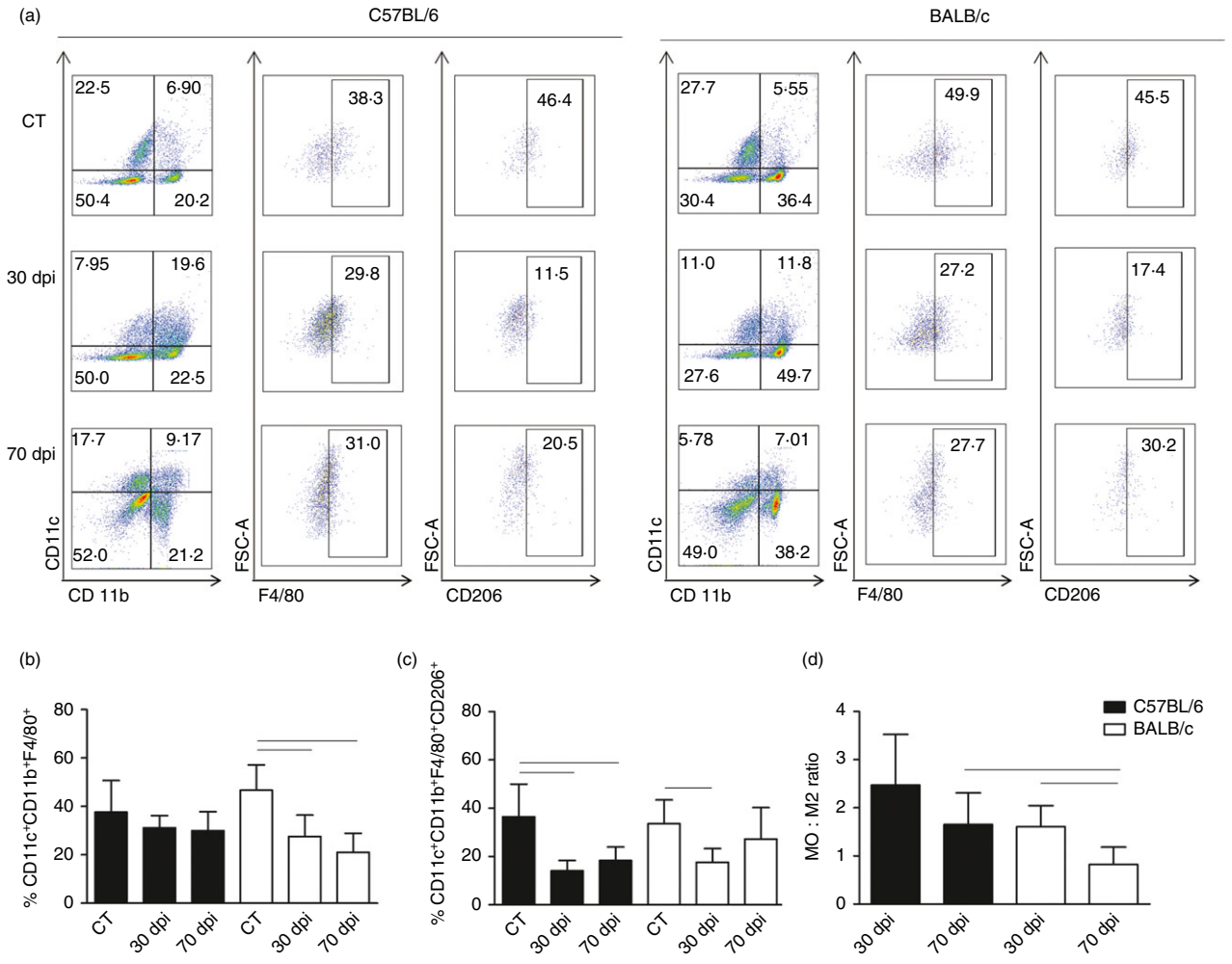

Figure 1. Chronically infected C57BL/6 mice exhibit a higher M0 : M2 ratio in the lungs than BALB/c mice. C57BL/6 (black bars) and BALB/c (white bars) mice were infected by the intratracheal route with 1×10^5 *Mycobacterium tuberculosis* bacilli or left uninfected (CT). Thirty days and 70 days after infection, lung cell suspensions obtained from digested lungs were stained with a monoclonal antibody against CD11c, CD11b, F4/80 and CD206. Macrophages were defined as $F4/80^+$ cells in the $CD11c^+$ $CD11b^+$ population. M2 macrophages were defined as $CD11c^+$ $CD11b^+$ $F4/80^+$ $CD206^+$ cells as represented in the gating strategy (a). The frequency of $CD11c^+$ $CD11b^+$ $F4/80^+$ cells (b) and $CD11c^+$ $CD11b^+$ $F4/80^+$ $CD206^+$ cells (c) and the $CD11c^+$ $CD11b^+$ $F4/80^+$ (M0): $CD11c^+$ $CD11b^+$ $F4/80^+$ $CD206^+$ (M2) ratio (d) were determined. Data are representative of two independent experiments and are presented as the mean ± SD (n = 19 animals for the CT groups and 9–10 animals for the infected groups). The bars show the differences ($P < 0{\cdot}05$) between experimental groups.

with counterpart BALB/c mice (see Supplementary material, Fig. S2a). The number of M2 macrophages ($CD11c^+$ $CD11b^+$ $F4/80^+$ $CD206^+$ cells) was also higher in the lungs of infected C57BL/6 and BALB/c mice (30 and 70 days) compared with their respective control groups (see Supplementary material, Fig. S2b). Additionally, the M0 : M2 ratio confirmed the significantly higher proportion of macrophages versus M2 macrophages when the C57BL/6 and BALB/c mice were compared at 70 days post-infection (see Supplementary material, Fig. S2c). The M0 : M2 ratio was lower in the lungs of C57BL/6 and BALB/c mice 70 days post-infection compared with their counterparts 30 days post-infection (see Supplementary material, Fig. S2c).

iNOS gene expression is increased in the lungs of chronically infected BALB/c but not C57BL/6 mice

To better characterize the macrophage subsets in the lungs of the mouse strains that generated different magnitudes of cellular immune responses during *M. tuberculosis* infection[27,29] we evaluated iNOS gene expression to characterize the pattern of M1 macrophages and Arg1 gene expression to characterize the pattern of M2 macrophages.

Because iNOS is expressed after cell activation, its expression was evaluated in the lungs of only the infected mouse strains. There was no difference in iNOS gene expression during infection (30 and 70 days post-infection) in the lungs of the C57BL/6 mice

(Fig. 2a). Although the iNOS expression was significantly lower in the lungs of the BALB/c mice at 30 days post-infection compared with the C57BL/6 mice at the same point, the iNOS expression level was significantly augmented at 70 days post-infection (Fig. 2a).

Arg1 gene expression showed the same pattern in the infected C57BL/6 and BALB/c mice. Infected C57BL/6 or BALB/c mice at 30 or 70 days post-infection exhibited significantly lower Arg1 gene expression in the lungs compared with their respective control groups. There was no difference in Arg1 expression when the C57BL/6 and BALB/c mice were compared (Fig. 2b). The iNOS : Arg1 ratio showed that there was no significant variation in the expression of both genes from 30 to 70 days post-infection in the lungs of the C57BL/6 mice. However, there was a significant change in the iNOS : Arg1 ratio in the BALB/c mice; this ratio was significantly increased in the lungs of chronically infected BALB/c mice compared with the level detected 30 days post-infection (Fig. 2c).

Next, we performed a correlation analysis between macrophages (M0) and iNOS expression and M2 macrophages (M2) and Arg1 expression to examine associations in the findings regarding macrophage subsets. We found no correlation between macrophages and iNOS gene expression for either the infected C57BL/6 or BALB/c mice (Fig. 3a). However, we found a significant positive correlation between M2 macrophages and Arg1 gene expression in the lungs of the infected BALB/c mice ($r = 0{\cdot}73$; $P = 0{\cdot}04$) but not the infected C57BL/6 mice (Fig. 3b). Moreover, we observed a significant positive correlation between M2 macrophages and the CFU numbers for the C57BL/6 ($r = 0{\cdot}81$; $P = 0{\cdot}01$) mice 70 days post-infection, but not for the infected BALB/c mice (Fig. 3c).

Chronically infected BALB/c exhibit high iNOS expression in vascular smooth muscle and pulmonary macrophages

Our findings show a lower frequency of $CD11c^+$ $CD11b^+$ $F4/80^+$ cells in the lungs of BALB/c mice and an increase in iNOS gene expression in the chronic phase of infection. Additionally, there was no correlation between $CD11c^+$ $CD11b^+$ $F4/80^+$ cells and iNOS expression for either the C57BL/6 or BALB/c mice. Therefore, we investigated whether iNOS was differentially expressed in the lungs of the infected BALB/c and C57BL/6 mice. Figure 4(a) shows the iNOS expression in the lungs of C57BL/6 mice 70 days post-infection. The upper panels show high iNOS expression in macrophages in the pulmonary parenchyma (left, arrows) and the bronchiolar epithelium (right, asterisks). The bottom panels (left and right) show a lack of iNOS expression in the vascular smooth muscle. Figure 4(b) shows the iNOS expression in the lungs of BALB/c mice 70 days post-infection. The upper panels (left and right) show high iNOS expression in macrophages in the pulmonary parenchyma (arrows) and the absence of iNOS expression in the bronchiolar epithelium. The bottom panels (left and right) show high iNOS expression in the vascular smooth muscle (asterisks).

M1 macrophages derived from the bone marrow of C57BL/6 or BALB/c mice exhibit no differential killing ability

To evaluate the function of M1 and M2 macrophages *in vitro*, bone-marrow-derived macrophages from C57BL/6 or BALB/c mice were differentiated into M1 or M2 macrophages and infected with *M. tuberculosis*. Phagocy-

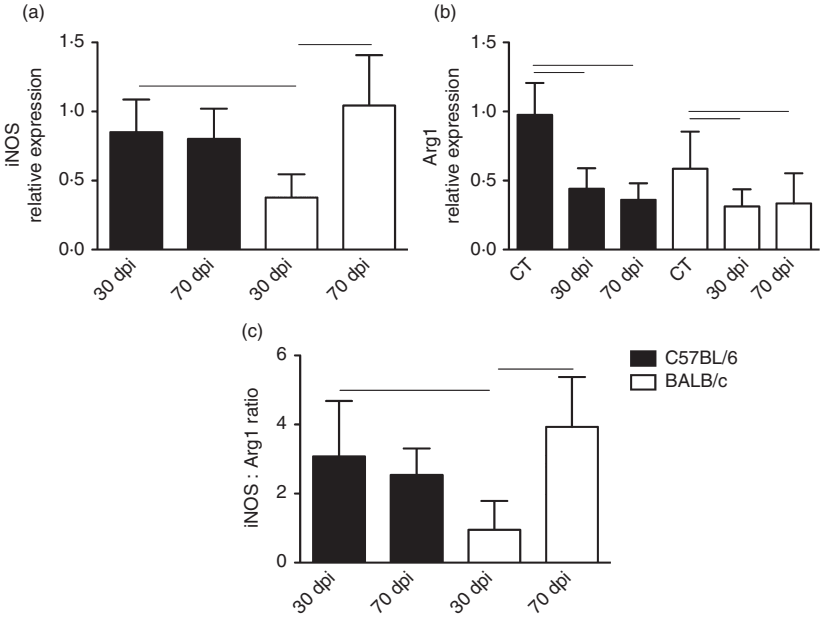

Figure 2. Inducible nitric oxide synthase (iNOS) gene expression is increased in the lungs of chronically infected BALB/c mice but not C57BL/6 mice. C57BL/6 (black bars) and BALB/c (white bars) mice were infected by the intratracheal route with 1×10^5 *Mycobacterium tuberculosis* bacilli or left uninfected (CT). Thirty and 70 days after infection, lung homogenates were obtained and iNOS (a) and Arg1 (b) gene expression was evaluated by real-time PCR. The iNOS : Arg1 ratio was determined (c). Data are representative of three independent experiments and are presented as the mean ± SD (n = 16–19 animals for the CT groups, n = 9 animals for the 30-day post-infection groups, and n = 14 animals for the 70-day post-infection groups). The bars show the differences ($P < 0{\cdot}05$) between experimental groups.

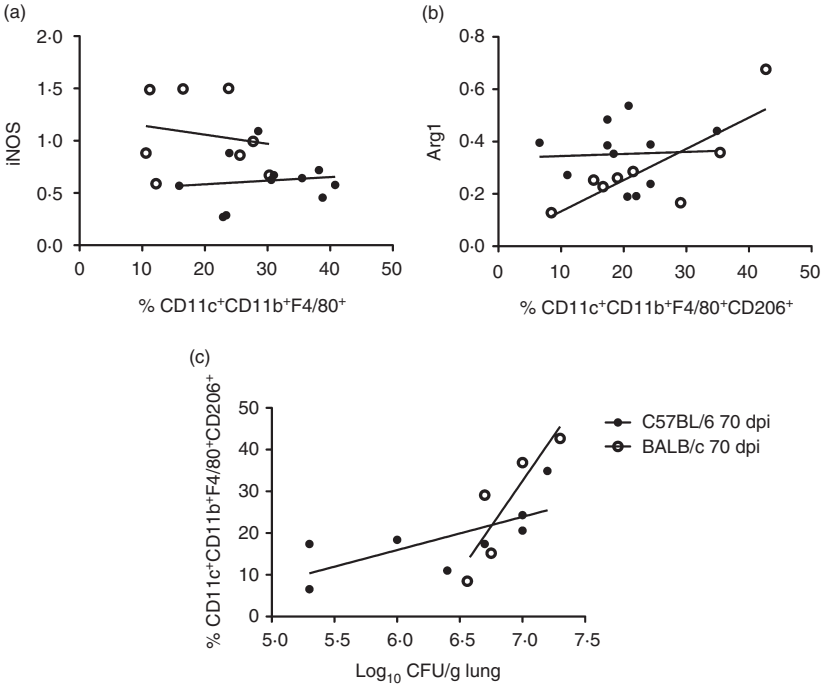

Figure 3. M2 macrophages correlate positively with lung colony-forming unit (CFU) counts. Inducible nitric oxide synthase (iNOS) and Arg1 expression and the CFU number were evaluated in the lung homogenates of C57BL/6 (black circles) and BALB/c (white circles) mice 70 days post-infection and were correlated with $CD11c^+$ $CD11b^+$ $F4/80^+$ cells or $CD11c^+$ $CD11b^+$ $F4/80^+$ $CD206^+$ cells. Correlation of iNOS expression and $CD11c^+$ $CD11b^+$ $F4/80^+$ cells (a). Correlation of Arg1 expression and $CD11c^+$ $CD11b^+$ $F4/80^+$ $CD206^+$ cells (b). Correlation of CFU number and $CD11c^+$ $CD11b^+$ $F4/80^+$ $CD206^+$ cells (c). Data are representative of two independent experiments and are presented as the mean ± SD.

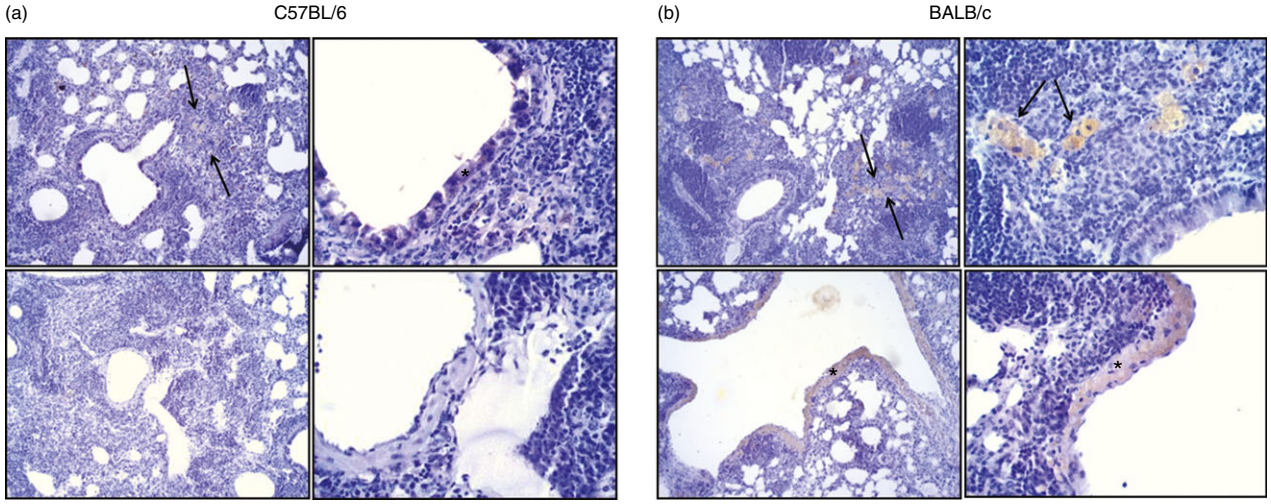

Figure 4. Chronically infected BALB/c mice exhibit high inducible nitric oxide synthase (iNOS) expression in macrophages and vascular smooth muscle. Expression of iNOS was evaluated in the lung sections of C57BL/6 (a) and BALB/c (b) mice 70 days post-infection. Expression of iNOS in the macrophages of the lung parenchyma (arrows) and in the bronchiolar epithelium (asterisks) of C57BL/6 mice (upper panels). The absence of iNOS expression in vascular smooth muscle (bottom panels). The iNOS expression in the macrophages of the lung parenchyma (arrows) and its absence in the bronchiolar epithelium of BALB/c mice (upper panels). iNOS expression in vascular smooth muscle (asterisks, lower panels).

tosis was evaluated after 4 hr, and the killing capacity was evaluated after 24 hr. First, we confirmed that macrophage differentiation conferred the M1 or M2 profile based on iNOS or Arg1 gene expression, respectively. M1 macrophages from the C57BL/6 and BALB/c mice exhibited higher expression of iNOS compared with Arg1. In contrast, M2 macrophages from the C57BL/6 and BALB/c mice exhibited higher expression of Arg1 compared with iNOS (Fig. 5a). Figure 5(b) shows that there was no difference in the phagocytosis capacity between the M1 or M2 subsets (C57BL/6 versus BALB/c mice). However, the M2 macrophages generated from the C57BL/6 and BALB/c mice exhibited a higher phagocytosis capacity compared with their M1 counterparts (Fig. 5b). M2 macrophages from the C57BL/6 mice were more permissive to bacterial replication than M1 macrophages (Fig. 5c). There was no difference in the killing ability between M1 and M2 macrophages generated from BALB/c mice (Fig. 5c).

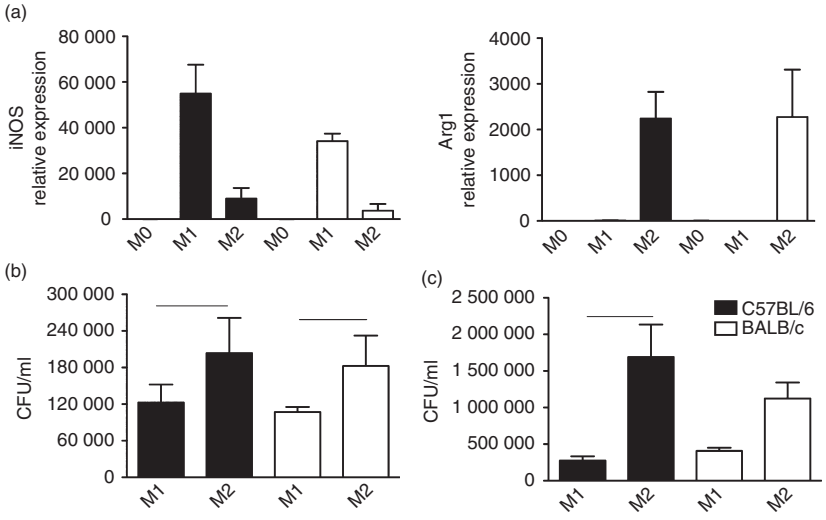

Figure 5. M1 macrophages derived from the bone marrow of C57BL/6 or BALB/c mice do not exhibit differential killing ability. Bone marrow-derived macrophages were differentiated into M1 or M2 macrophages (a) and infected with *Mycobacterium tuberculosis* (multiplicity of infection 5). Four (phagocytosis, b) or 24 hr (killing, c) cell lysates were serially diluted, and the colony-forming unit (CFU) counts were determined after 28 days. Data are representative of two independent experiments and are presented as the mean ± SD ($n = 5$ C57BL/6 or BALB/c mice). The bars show the differences ($P < 0{\cdot}05$) between macrophage subsets.

Increased numbers of M2 macrophages render C57BL/6 mice more susceptible to *M. tuberculosis* infection

To ascertain the role of M1 and M2 macrophages *in vivo*, bone-marrow-derived macrophages from C57BL/6 or BALB/c mice were differentiated into M1 or M2 macrophages *in vitro*; then, 2×10^6 cells were transferred by intratracheal injection into the respective mouse strain 60 days post-infection. Additionally, one group of mice from each mouse strain received bone-marrow-derived macrophages (M0). Ten days after the cell transfer (70 days post-infection), the lungs were collected for the CFU assay and histopathological analysis.

We confirmed the iNOS and Arg1 gene expression levels in the M0, M1 and M2 macrophages differentiated from the bone marrow precursors, used for the cell transfer (data not shown). We also confirmed that the macrophage subsets reached the lungs by evaluating CFSE-stained M1 or M2 macrophages 24 hr after the intratracheal cell transfer (Fig. 6a).

The M2 cell transfer significantly increased the susceptibility of infected C57BL/6 mice, whereas the M1 cell transfer did not affect the bacterial load in the lungs of these mice (Fig. 6b). The M1 or M2 cell transfer did not affect significantly the susceptibility of the infected BALB/c mice (Fig. 6c).

The analysis of lung sections from C57BL/6 mice 70 days post-infection showed an extensive inflammation characterized by a higher number of lymphocyte follicles mostly in the perivascular and peribronchial areas. Moreover, the inflammation was extensive in the lung parenchyma of the infected C57BL/6 mice (Fig. 7a). The M1 cell transfer to infected C57BL/6 mice induced a lobular inflammation with a scarce number of lymphocyte follicles (Fig. 7b) compared with the infected C57BL/6 mice that did not undergo M1 cell transfer (Fig. 7a). The M2 cell transfer to infected C57BL/6 mice resulted in mild inflammation characterized by an accumulation of macrophages (Fig. 7c) compared with the infected C57BL/6 mice that did not undergo cell transfer (Fig. 7a). The M0 cell transfer (data not shown) induced a pattern of inflammation similar to the pattern found in the lungs of C57BL/6 mice that received M1 cell transfer.

A mild inflammation characterized by the presence of lymphocyte follicles and macrophages was found in the lung sections of BALB/c mice 70 days post-infection (Fig. 7d) compared with the C57BL/6 mice at the same time-point (Fig. 7a). The M1 cell transfer to infected BALB/c mice was associated with an increase in the number of lymphocyte follicles predominantly in the peribronchial area and an intense accumulation of macrophages (Fig. 7e) compared with BALB/c mice that did not undergo the cell transfer (Fig. 7d). Figure 7(f) shows that the M2 cell transfer to infected BALB/c mice conferred a pattern and magnitude of inflammation similar to those observed in the BALB/c mice that did not undergo cell transfer (Fig. 7d). However, the inflammation in the lungs of the infected BALB/c mice transferred with M2 macrophages was characterized by a lower lymphocyte influx and higher magnitude of macrophages (Fig. 7f) than the infected BALB/c mice that received M1 macrophages (Fig. 7e). The M0 cell transfer (data not shown) induced a pattern of inflammation similar to that found in the lungs of BALB/c mice that received M1 cell transfer.

Discussion

Classically, the type 1 response (M1 and Th1) is associated with protection against tuberculosis.[33] However, patients with tuberculosis experience respiratory difficulties as a consequence of the excessive inflammatory response generated by bacillus persistence. Therefore, in

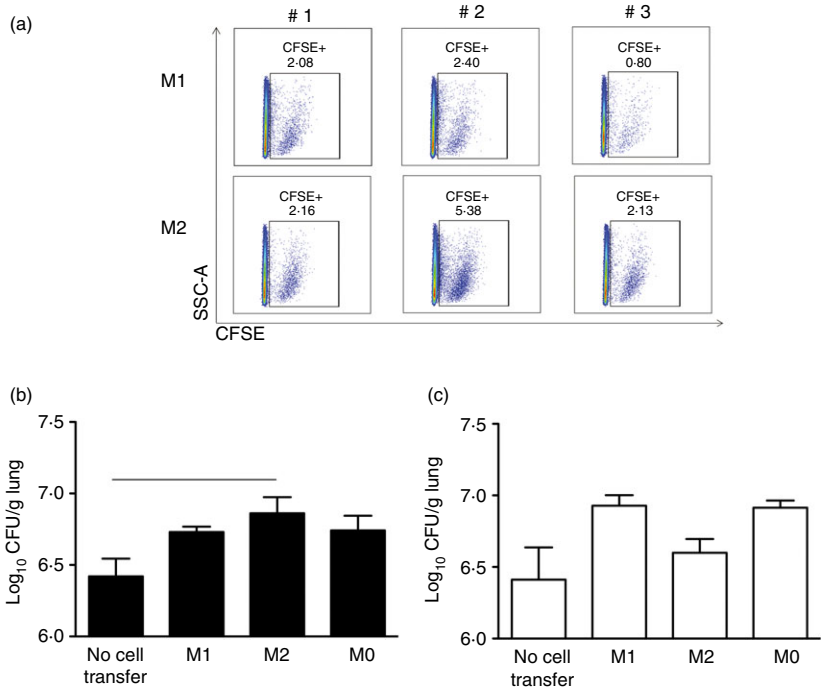

Figure 6. Increased M2 macrophages render C57BL/6 mice more susceptible to *Mycobacterium tuberculosis* infection. Bone marrow-derived macrophages from C56BL/6 or BALB/c mice were differentiated into M1 or M2 macrophages; then, 2×10^6 cells were transferred by intratracheal injection to the respective mouse strain at 60 days of infection. Ten days after cell transfer (70 days of infection), the lungs were collected for the colony-forming unit (CFU) assay. CFSE-stained M1 or M2 macrophages in the lungs 24 hr after the intratracheal cell transfer (a). CFU counts in the lungs of C57BL/6 (b) and BALB/c (c) mice. Data are presented as the mean ± SD (n = 4–6 animals/group). The bars show the differences ($P < 0{\cdot}05$) between experimental groups.

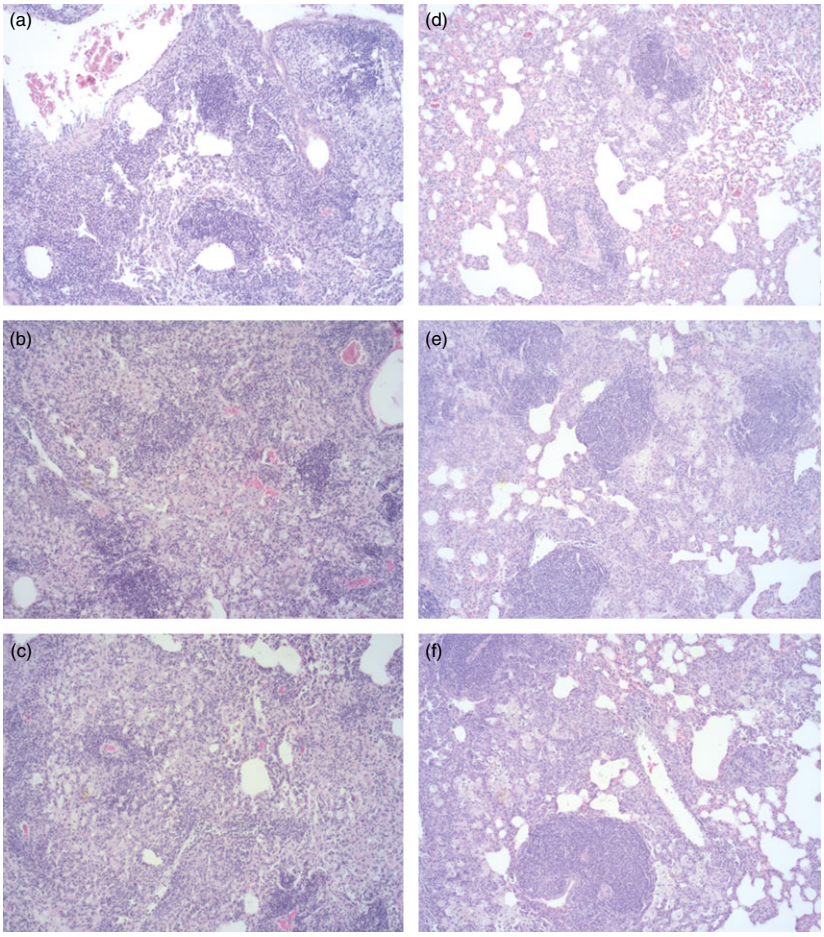

Figure 7. M2 cell transfer reduces inflammation in the lungs of C57BL/6 mice whereas M1 cell transfer increases the lymphocyte follicles in the lungs of BALB/c mice. Bone marrow-derived macrophages from C56BL/6 or BALB/c mice were differentiated into M1 or M2 macrophages; then, 2×10^6 cells were transferred by intratracheal injection to the respective mouse strain at 60 days of infection. Ten days after cell transfer (70 days of infection), the lungs were collected for histopathological analysis. Lung section of a C57BL/6 mouse with no cell transfer (a). Lung section of a C57BL/6 mouse after M1 cell transfer (b). Lung section of a C57BL/6 mouse after M2 cell transfer (c). Lung section of a BALB/c mouse with no cell transfer (d). Lung section of a BALB/c mouse after M1 cell transfer (e). Lung section of a BALB/c mouse after M2 cell transfer (f).

addition to generating an effective immune response to clear the bacteria, a balanced inflammatory response is required for the host to avoid lung damage.[34,35]

In this study, we showed a higher M0 : M2 ratio in the lungs of chronically *M. tuberculosis*-infected C57BL/6 mice that also presented a higher inflammation, compared with BALB/c mice. These findings suggest that the host genetic background and the microenvironment induced by the infection impair the differentiation of M2 macrophages in the lungs of C57BL/6 mice and impair the differentiation of inflammatory macrophages in the lungs of BALB/c mice. However, there was no difference in the M1 macrophage killing ability between the C57BL/6 and BALB/c mice. In accordance with our initial hypothesis, we expected to find a high frequency of macrophages in the lungs of C57BL/6 mice followed by high iNOS expression based on the high magnitude of the Th1 immune response in *M. tuberculosis*-infected C57BL/6 mice.[27,29] Moreover, we hypothesized that M1 macrophages derived from the bone marrow of C57BL/6 mice would exhibit a high ability to kill bacilli compared with the M1 macrophages from BALB/c mice. Conversely, we expected to find a high frequency of M2 macrophages in the lungs of BALB/c mice and speculated that this M2 subset would be more permissive for bacterial replication.

Our findings were different from our initial hypothesis. Compared with the macrophage subset in the lungs of naive C57BL/6 mice, *M. tuberculosis* infection did not change the frequency of these cells and reduced the frequency of M2 macrophages. Moreover, iNOS expression was invariable between 30 and 70 days post-infection. The opposite result was observed in the lungs of infected BALB/c mice 70 days post-infection: a significant decrease in the frequency of macrophages followed by an unchanged frequency of M2 macrophages compared with their uninfected counterpart group. However, there was an increase in iNOS expression 70 days post-infection. In a previous study, we showed a higher concentration of NO in the lungs of BALB/c mice compared with C57BL/6 mice during the chronic phase of infection.[27] In the current study, no correlation was found between macrophages and iNOS, whereas M2 macrophages were positively correlated with Arg1 expression during the chronic phase of infection in the BALB/c mice. We showed increased expression of iNOS in the pulmonary parenchyma. In addition, the BALB/c mice exhibited iNOS expression in the vascular smooth muscle. However, determining whether iNOS expression co-localizes with $CD11c^+$ $CD11b^+$ $F4/80^+$ cells requires further investigation.

Therefore, our first partial conclusion is that the microenvironment characterized by a strong Th1 immune response in the lungs of infected C57BL/6 mice[27,29] hampers the differentiation of M2 macrophages. In contrast, the mixed immune response found in the lungs of chronically infected BALB/c mice[27,28] hampers the differentiation of inflammatory macrophages. We suggest that dendritic cell subsets play a critical role in the magnitude of inflammation induced in the lungs of infected C57BL/6 mice and this affects the outcome of the infection in this mouse strain compared with BALB/c mice. The $CD11c^+$ $CD103^+$ cell subset in the lungs of infected C57BL/6 mice induced a higher frequency of IFN-γ-producing $CD4^+$ cells than in infected BALB/c mice.[29] Based on the current findings reported here, we suggest that activation of $CD11c^+$ $CD103^+$ cells is important for the induction of the type 1 inflammatory response. In addition, at 70 days of infection, infected BALB/c mice exhibit a mild inflammation, probably as a consequence of mixed pattern of cellular immune response, low number of $CD11c^+$ $CD103^+$ cells and high number of $CD11c^+$ $CD11b^+$ cells as previously characterized.[29] Infected BALB/c mice produced higher levels of IL-10 and reduced levels of IFN-γ and exhibited a higher number of $Foxp3^+$ cells in the lungs as the infection progressed.[27]

Our second partial conclusion is that the iNOS expression does not play an essential role in *M. tuberculosis* infection. Neither the unchanged iNOS expression levels 30 and 70 days post-infection in the C57BL/6 mice nor the increase in iNOS expression in the BALB/c mice was associated with the CFU number. Indeed, the exact role of NO in the protection against *M. tuberculosis* infection is unclear. iNOS deficiency renders infected C57BL/6 mice more susceptible to infection.[15,16] However, little is known about iNOS deficiency in BALB/c mice. At present, the production of NO by human macrophages promotes growth of mycobacteria.[36,37]

Inflammatory macrophages such as M1 macrophages cooperate to increase the magnitude of the Th1 immune response. Despite being unable to ascertain that the $CD11c^+$ $CD11b^+$ $F4/80^+$ cells (which we described here as macrophages) were M1 macrophages, the magnitude of pulmonary inflammation in the C57BL/6 mice was characterized by an accentuated prevalence of lymphocyte follicles compared with the macrophage infiltrate.

The characterization of macrophages in the lungs of infected C57BL/6 mice based on the CD11c and CD11b markers showed a difference between alveolar macrophages and small macrophages.[38] In our hands, we could not distinguish macrophage subsets in the lungs based on the fluorescence intensity of these markers. However, we used both markers plus the F4/80 marker to define the macrophages in the lungs. The use of these markers (CD11c, CD11b and F4/80) plus CD206 allowed us to discriminate macrophages from M2 macrophages. We used this strategy to study macrophage subsets from the perspective of the host infected with *M. tuberculosis*. One study showed that BALB/c mice (reported as susceptible) infected with *Mycobacterium intracellulare* presented more

suppressive macrophages that exhibited the ability to generate intermediate levels of reactive oxygen species compared with CBA/JN mice (reported as resistant).[39]

Despite finding no difference in the M1 macrophage killing ability between the C57BL/6 and BALB/c mice, M2 macrophages obtained from C57BL/6 mice were more permissive to mycobacterial growth than M1 macrophages, whereas there was no difference in the killing ability between M1 and M2 macrophages generated from BALB/c mice. In agreement with *in vitro* results and positive correlation between $CD11c^+$ $CD11b^+$ $F4/80^+$ $CD206^+$ cells and CFU number, the *in vivo* M2 cell transfer to infected C57BL/6 mice conferred higher susceptibility to these mice followed by a lower magnitude of inflammation compared with their counterparts that did not undergo cell transfer. In contrast, the M2 cell transfer did not render infected BALB/c mice more susceptible to infection compared with their counterparts that did not undergo cell transfer.

From the perspective of the infected C57BL/6 mice (based on the macrophage number and M2 frequency, M0 : M2 ratio, and M2 cell transfer), our findings confirmed previous reports that M2 macrophages played a deleterious role in *M. tuberculosis* infection.[23–25] We showed for the first time that the genetic background was associated with the frequency and function of these cells during *M. tuberculosis* infection. There are genetic studies showing the association between polymorphisms of CD206[40] or IL-10[41] with tuberculosis and the association of an iNOS locus and protection against tuberculosis.[15] These studies associate indirectly the genetic repertoire with macrophages and tuberculosis.

We suggest that the magnitude of the Th1 immune response is directly associated with the different M0:M2 ratios found in the C57BL/6 and BALB/c mice during the chronic phase of infection. Moreover, the histopathological analysis showed a higher magnitude of inflammation in the lungs of the C57BL/6 mice compared with the BALB/c mice 70 days post-infection. Therefore, we suggest that the magnitude of the type 1 response is associated with bacterial tolerance, although it may result in tissue damage. In contrast, the mixed type 1 and type 2 responses in BALB/c mice are associated with tolerance against tissue damage although they are detrimental for bacterial tolerance.

To reinforce the discussion of bacterial tolerance, tissue tolerance and the magnitude of inflammation, the increase in the M0 or M1 subsets in the lungs of BALB/c mice did not render these animals more resistant to infection, suggesting that although the M1 cell transfer increased the number of lymphocyte follicles, these lymphocytes could be down-modulated by the mixed pattern of type 1 and type 2 response described in the BALB/c mice.[27,29]

In summary, we suggest that there is a balance between the immune response, inflammation and the bacterial load in the lungs of infected C57BL/6 mice. A lower magnitude Th1 response in BALB/c mice favours the maintenance of M2 macrophages that can reduce the pulmonary inflammatory response. However, the mixed inflammatory and non-inflammatory responses may favour bacterial spreading.

Finally, we conclude the following: (i) the genetic background affects the expansion of macrophage subsets in the lungs of *M. tuberculosis*-infected hosts; (ii) the genetic background does not affect the killing ability of M1 macrophages *in vitro*; (iii) the proportion of M0 : M2 appears to be more critical than the iNOS-dependent killing ability for the infection fate in genetically different hosts. In a microenvironment with a mixed immune response, the down-regulation of mediators that disable the cellular immune response seem to be more essential than the up-regulation of inflammation.

These findings reinforce the previous premise that the development of new forms of treatment for tuberculosis should consider the genetic background of the host to improve bacterial clearance without inducing inflammation or modulating the magnitude of the inflammation.

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Disclosures

The authors declare that they have no competing interests to disclose.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Chronically *Mycobacterium tuberculosis*-infected C57BL/6 mice are more resistant than BALB/c mice.

Figure S2. C57BL/6 (black bars) and BALB/c (white bars) mice were infected as described in Fig. 1.